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Biosynthesis of Pseudouridine in Transfer Ribonucleic Acid*

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SUMMARY

An enzyme that catalyzes the formation of specific pseudouridine residues in the anticodon region of tRNA has been characterized in extracts of *Salmonella typhimurium* and *Escherichia coli*. It has previously been shown that *hisT* regulatory mutants of *S. typhimurium* lack the pseudouridine modifications in the anticodon region of histidine and several other tRNA species. This has permitted the development of a simple radiochemical assay for the modification enzyme, based on the release of tritium from *hisT* tRNA isolated from cells grown in [5-^{3}H]uridine. This enzyme activity is absent from *hisT* mutants and is relatively heat-labile in extracts from temperature-sensitive *hisT* mutants, which suggests that the modification enzyme itself is the primary gene product of the *hisT* gene.

Among the various minor nucleosides present in tRNA, pseudouridine is the most common. All known tRNA sequences contain a pseudouridine (ψ) residue in the "TψCPu" side-arm loop (1) except for yeast $tRNA_F^{Met}$ (2) and a $tRNA^{Gly}$ not used in protein synthesis (3). Pseudouridine residues have been located in other regions of the primary structure of tRNA. In many tRNA species from *Escherichia coli* and *Salmonella typhimurium* 1 or 2 pseudouridine residues have been found in the stem and loop of the anticodon region (4–10).

During the study of the regulation of histidine biosynthesis in *S. typhimurium*, we discovered that *hisT* mutants (11), one of the six classes of mutants derepressed for the histidine operon (12), contained a $tRNA^{His}$ lacking 2 pseudouridine residues which are normally present in the anticodon region of wild type $tRNA^{His}$ (13). In contrast, the pseudouridine in the TψCPu loop was present in both the wild type and mutant $tRNA^{His}$ (12). We have also presented evidence that, as a consequence of the *hisT* mutation, the chromatographic properties of several other species of tRNA are altered without greatly affecting the function of these tRNAs in protein synthesis (13).

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Such findings strongly implied that the *hisT* gene product might be an enzyme that catalyzes the formation of pseudouridine in the anticodon region of specific tRNA species. If this were true, then bulk tRNA from a *hisT* mutant should contain a significant number of residues specifically capable of modification to pseudouridine by enzymes from wild type strains. This situation is analogous to the ability of wild type extracts to modify the submethylated species of tRNA that are synthesized by mutants with defective methylation enzymes (14), or the submethylated and subthiolated tRNA produced by relaxed amino acid auxotrophs (15, 16).

In this paper, we identify an enzymatic activity in wild type strains of *S. typhimurium* and *E. coli* which can modify specific uridine residues of bulk *hisT* tRNA. This activity is not detectable in extracts derived from *hisT* strains and is heat-labile in temperature-sensitive *hisT* strains. These findings offer further evidence that this pseudouridylation enzyme represents the *hisT* gene product.

MATERIALS AND METHODS

Chemicals—Chemicals used for the growth of the cells and for the extraction and analysis of nucleic acid were of reagent grade or otherwise of the highest available purity. [5-^{3}H]Uridine (20,000 mCi per mmole), [6-^{3}H]uridine (20,000 mCi per mmole), and $^{32}P_i$ (carrier-free) were purchased from Schwarz-Mann. DEAE-cellulose paper (Machrey, Nagel and Co.), T1 ribonuclease (Calbiochem), and bovine pancreatic RNase A (Worthington) were purchased from commercial sources.

Bacterial Strains—Isogenic *hisT* (TA253) and wild type *S. typhimurium* LT-2 (TA265) were constructed by George Chang (Univ. of California, Berkeley) by transducing *hisT1504* and *hisT*$^+$ into an *aroD purF* double auxotroph (11).

Growth of Cells and Preparation of Extracts—Cells were grown in Vogel-Bonner minimal E medium with 0.2% glucose (17) at 37°, harvested in late log phase (A = 1.0 at 650 nm), and kept frozen for several months. Cells were suspended in 2 volumes of buffer containing 10 mM Tris-HCl buffer, pH 7.8, 1 mM EDTA, 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, and 10% glycerol (v/v), and were sonicated three times for 30 s each. After an initial centrifugation at 30,000 × g for 30 min, the supernatant was respun at 300,000 × g for 90 min. Such extracts were used directly for all of the work reported here.

Preparation of ^{3}H-labeled tRNA—Cells were grown in 500 ml of the minimal glucose medium (17) containing 10 mCi of [5-^{3}H]-uridine. When they reached the late log phase, they were centrifuged at low speed, suspended in 4 ml of 10 mM Tris-HCl, pH

7.5, and 10 mM $MgCl_2$ (Buffer A), disrupted by sonication, then centrifuged at 30,000 × *g* for 30 min. The supernatant was respun at 100,000 × *g* in a Spinco SW 39 rotor for 4 hr. The supernatant was extracted with distilled phenol saturated with Buffer A, and the tRNA was further purified as previously described (18). The specific activity averaged 10 μCi per A_{260} unit of tRNA. Of the incorporated radioactivity, 63% was found in CMP and 37% in UMP and UMP derivatives, on the basis of alkaline and enzymatic hydrolysis of the product. Both methods of hydrolysis gave identical results.

Preparation of [^{32}P]tRNA and Nucleotide Analysis—Preparation of [^{32}P]tRNA was done according to the procedure described by Singer and Smith (10). After enzymatic modification of the tRNA (see below), the reaction was stopped by the addition of 1 volume of twice distilled phenol, neutralized with Buffer A. Carrier yeast tRNA (50 μg) was added and the samples were shaken for 1 hr at 0°. Approximately 80% of the aqueous phase was carefully removed with a micropipette and the operation was repeated after addition of 0.1 ml of water to the remaining sample. The aqueous phases were combined and NaCl was added (final concentration, 0.5 M), followed by 2.5 volumes of ethanol. The samples were left at −20° overnight and were centrifuged in a clinical centrifuge. The small pellets were resuspended in 10 μl of water and subjected to alkaline hydrolysis, as described earlier (10). The hydrolysates were resolved initially by electrophoresis on DEAE-cellulose paper in pyridine-acetate buffer, pH 3.5, for 3 hours at 2500 volts. UMP and ψMP migrate together in this system. After radioautography, the spot containing the two nucleotides was eluted, taken to dryness and chromatographed on Whatman No. 1 paper, using the solvent, isopropyl alcohol-HCl-water (75:17:1, v/v/v). The UMP and ψMP spots were well separated in this system (19) and after radioautographic identification, they were cut from the paper and counted by Cerenkov radiation in a Nuclear-Chicago Mark II scintillation spectrometer.

Enzyme Assays—The tritium release assay for pseudouridylation activity was carried out as follows. The incubation mixtures, 0.1-ml total volume, contained 100 mM glycine-NaOH buffer, pH 9.0; 20 mM $MgCl_2$; 0.02 A_{260} unit of tritiated tRNA; and enzyme. The reaction was started by addition of enzyme, and the samples were incubated at 37°. The reaction was terminated by addition of 1 ml of 12% activated charcoal (Norit A) in 0.1 N HCl (w/v). The samples were left at room temperature for 20 min, with occasional stirring, and then were filtered onto membrane filters (0.45-μ pore size). The filtrate was collected directly into scintillation vials and, after addition of 10 ml of Bray's solution, was counted in a scintillation spectrometer with an over-all efficiency of 15%. Control incubation mixtures from which the enzyme had been omitted (Table I) or which were sampled at zero time gave blanks in the range of 40 to 60 cpm. The data in most of the figures shown have been corrected for these blank values.

Pseudouridylate synthetase was assayed according to Breitman (20).

RESULTS

Identification and Specificity of Enzyme Activity—During the synthesis of pseudouridine, the formation of the C—C bond between C_5 of the pyrimidine ring and C_1 of the ribose must be accompanied by the release of a proton into the medium (Fig. 1). The availability of pyrimidine derivatives, tritiated at C_5 of the ring, has permitted the development of highly sensitive tritium release assays for a number of enzyme reactions involving a similar proton release (20–24). We have used such a tritium release procedure, starting with a labeled tRNA substrate from a *hisT* mutant for the detection of the pseudouridylation activity.

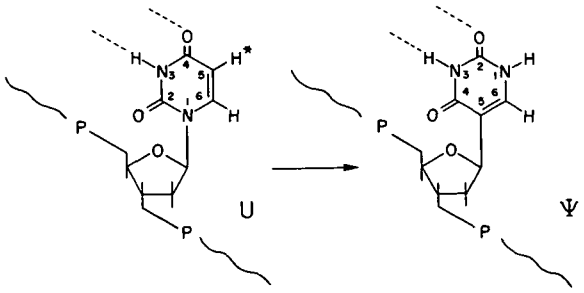


FIG. 1. Tritium release during formation of tRNA pseudouridine residues. The scheme illustrates a possible mechanism for pseudouridine formation. As shown, the generation of a bond between C_5 of the pyrimidine ring and C_1 of the pentose necessarily involves loss of the hydrogen from the C_5 of the ring.

TABLE I

Release of ^{3}H in pseudouridylation assay

The incubation mixture contained 40 pmoles of tritiated tRNA (1200 cpm per pmole) from the wild type strain or mutant *hisT1504;* 100 mM glycine-NaOH buffer, pH 9.0; 20 mM $MgCl_2$; and 200 μg of enzyme protein in a final volume of 0.1 ml. The reaction was carried out for 30 min at 37° and terminated, and the activity was measured as described under "Materials and Methods." The counts per min released are for the entire reaction mixture.

Enzyme source	Wild-type tRNA: Experiment 1	*HisT* tRNA	
		Experiment 1	Experiment 2
	cpm	*cpm*	
No enzyme	47	51	22
HisT1504	45	56	31
HisT1309	51	54	
Wild type	57	435	573
Wild type (zero time)			34
Escherichia coli B			560

The tRNA, tritiated at C_5 of the pyrimidine ring, was prepared from a *hisT* mutant of *S. typhimurium* grown in the presence of [5-^{3}H]uridine (see "Materials and Methods"). In the presence of a pseudouridylation activity, tritium should be released into the medium at a rate proportional to the enzyme activity. The data shown in Table I demonstrate the presence of such an activity in extracts from wild type *S. typhimurium.* Under the same conditions, no tritium release occurred with labeled wild type tRNA, which indicates that this tRNA lacked modifiable pyrimidine residues. It was also possible to detect this activity in extracts from *E. coli* B which had about the same activity as found in *S. typhimurium.*

The tritium release activity was also tested with extracts from two independently isolated *hisT* mutants: *hisT1504*, the source organism for the labeled tRNA and the strain used for determination of the $tRNA^{His}$ sequence (13); and *hisT1309*, an amber mutant in the same gene (11). Tritium release activity was undetectable in extracts from both *hisT* strains.

With *hisT* tRNA labeled at C_5, the rate of tritium release was linear for 5 or 10 min and continued for 30 min or more under the conditions shown in Fig. 2. On the other hand, tRNA from the same mutant, but tritiated at the C_6 position of the pyrimidine residues, did not release any tritium, even after extended

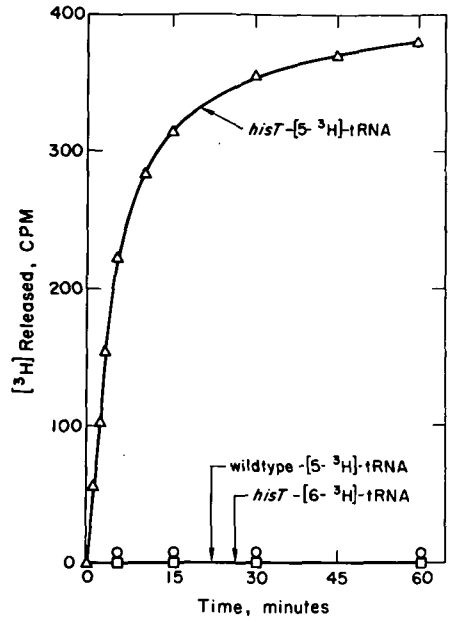


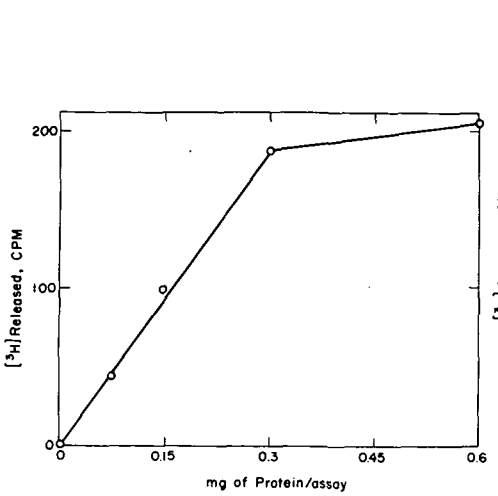
FIG. 2. Rate and specificity of tritium release. Assay conditions were identical with those of Table I, except that 100 μg of the protein were used.

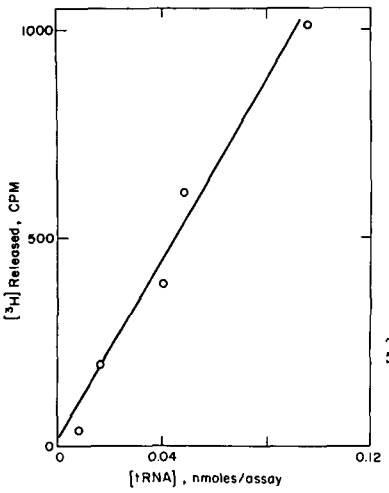
reaction of about 0.4 mole of uridine per mole of tRNA.[1] This value may not necessarily represent the maximum number of modifiable sites in the tRNA, since the crude extracts used in the experiments might have contained inhibitors, nucleases, or other contaminants affecting the plateau. This aspect of the reaction deserves further examination with purified enzymes.

In Fig. 5, the initial rates of tritium release are plotted as a function of tRNA concentration. From the Lineweaver-Burk plot (see *inset*), an apparent K_m of approximately 10^{-6} M is obtained. This value is a maximum estimate, since it is based on the assumption that every tRNA molecule is a substrate for the enzyme. However, since not all molecules of tRNA contain pseudouridine modifications in the anticodon region (1), the affinity of the enzyme for the specific tRNA substrate is probably even greater than the apparent K_m would indicate.

At high concentrations of tRNA, there appears to be a small divergence from ideal Lineweaver-Burk kinetics (see *inset*, Fig. 5). This could indicate that the substrate tRNA mixture contained nonmodifiable sequences or configurations which competed for the enzyme site or which interfered with the enzyme activity. This feature deserves further examination.

Other reaction requirements are given in Figs. 6 and 7. The pH dependence of the reaction (Fig. 6) showed a sharp optimum at 9.0. The reaction also showed an absolute requirement for





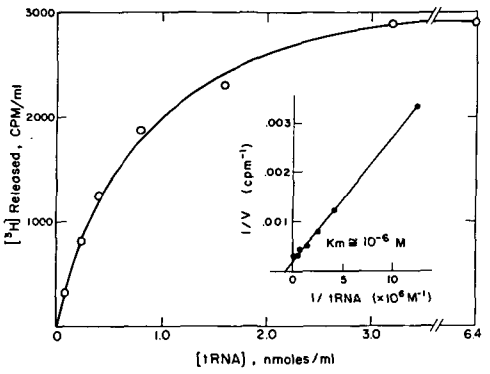


FIG. 3 (*left*). Rate of tritium release as a function of enzyme concentration. Reaction conditions were those shown in Table I. The incubation was carried out for 3 min at 37°.

FIG. 4 (*center*). Relationship between the plateau of tritium release and tRNA concentration. The assay conditions were those given in Fig. 2. The incubations were carried out for 60 min in order to achieve a plateau of tritium release (see Fig. 2).

FIG. 5 (*right*). Initial rate of tritium release as a function of *hisT* tRNA concentration. The reaction conditions were those of Fig. 2. The incubation time was 3 min. The V values shown in the *inset* refer to counts per min of ^{3}H released.

incubation (Fig. 2). This provides further support for the specificity of the reaction and suggests that the mechanism does not involve any randomization of hydrogen atoms at carbon 6 of the pyrimidine ring.

Kinetic Features of the Enzyme Reaction—Fig. 3 depicts the tritium release activity as a function of the enzyme concentration. With *hisT* tRNA at 0.4 nmole per ml, the rate of tritium release was a linear function of the concentration of enzyme, up to a saturating level of 3 mg per ml of protein.

The maximum extent of tritium release was determined by prolonged incubation with subsaturating levels of enzyme and was directly proportional to the initial concentration of tRNA (Fig. 4). From the specific activity of the tRNA and its uridine content, we estimate that the plateau levels correspond to the

Mg^{2+} (data not shown) and was optimal above a Mg^{2+} concentration of 1.0 mM.

The activity was highly sensitive to increased salt concentration (Fig. 7). At 65 mM NaCl, the activity was only 50% of the optimum rate. However, full enzyme activity was maintained

[1] This value has been determined as follows. The concentration of tRNA was calculated from an assumed absorbance of 20 A_{260} per mg, and an average molecular weight of 25,000. Accordingly, 0.02 A_{260} unit was equivalent to 1 μg or 40 pmoles of tRNA. Each picomole of *hisT* tRNA was assumed to contain 14 pmoles of uridine (25), which accounted for 37% of the ^{3}H, as determined by direct experiment (see "Materials and Methods"). With a specific activity of 1,200 cpm per pmole of tRNA, the modification of 1 pmole of uridine would correspond to the release of 1,200 × 0.37/14 = 31.7 cpm of ^{3}H.

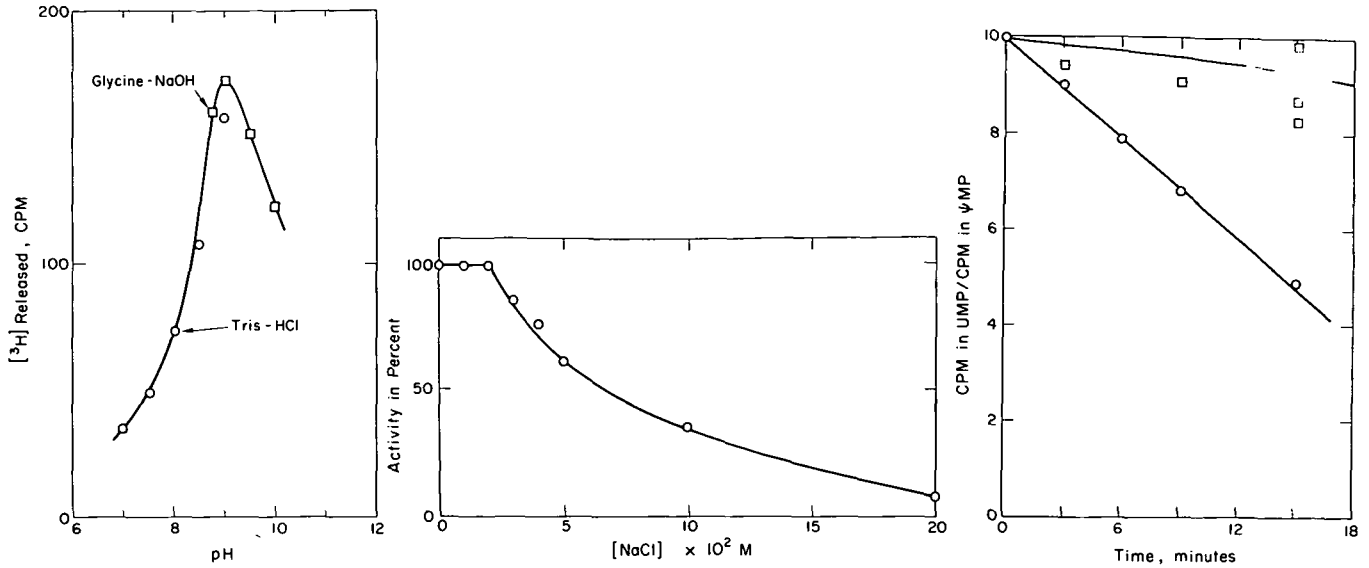


FIG. 6 (*left*). pH Dependence of tritium release. The standard assay procedure was used, but the buffer pH was varied as shown, at a concentration of 100 mM. The assay mixtures contained 20 μg of enzyme protein and were incubated for 5 min.

FIG. 7 (*center*). Effect of salt concentration on rate of tritium release. The reaction conditions were those given in Table I, except that 20 μg of enzyme protein were used, and NaCl was added to the final concentrations shown. The incubation was for 5 min at 37°.

FIG. 8 (*right*). Formation of tRNA pseudouridylate during incubation of *hisT* tRNA with enzyme extracts. The incubation mixtures contained 100 mM glycine buffer, pH 9.0; 20 mM $MgCl_2$; 100 pmoles per ml of ^{32}P - labeled *hisT* tRNA and 500 μg per ml of enzyme protein from either the wild type strain or from strain *hisT1504*. At the designated time points, 0.1-ml samples were removed and mixed with an equal volume of neutralized phenol. Carrier yeast tRNA was added and the nucleic acids were isolated, hydrolyzed, and subjected to nucleotide analysis as described under "Materials and Methods."

when the salt concentration was restricted to 20 mM or less. The inhibition by salts appeared to be nonspecific, since $(NH_4)_2SO_4$, NH_4Cl, KCl, and NaCl were equally inhibitory at a concentration of 100 mM.

The extract was readily desalted by passage through Sephadex G-50, but the activity was sometimes lost after dialysis. The preparation could be stored for at least 10 days in 10% glycerol with no loss of activity, but freezing and thawing led to severe losses.

Characterization of Pseudouridylate Formation by Enzyme Extracts—In order to verify the occurrence of the pseudouridylate modification, it was necessary to demonstrate that the action of the enzyme led to the disappearance of uridylate or cytidylate residues, with the concurrent appearance of pseudouridylate. This was carried out by incubation of [^{32}P]tRNA from the *hisT* mutant with wild type enzyme extract. As a control, the *hisT* tRNA was treated with homologous enzyme from the same *hisT* strain. The results are shown in Fig. 8. In order to normalize the results for variable losses during the analytical steps, the activity is expressed in terms of the ratios of UMP:ψMP during the course of the reaction. For bulk *hisT* tRNA, this ratio is equal to 10, whereas it is equal to 5 for wild type tRNA. When the *hisT* tRNA was treated with enzyme from wild type cells, this ratio decreased to 5 within a 15-min incubation period (Fig. 8). In the control incubation, the ratio changed only slightly, from 10 to approximately 9, during the same time course.

It is doubtful whether the small change in UMP:ψMP ratio in the control incubation has any significance, particularly since such homologous reaction mixtures were inactive in the tritium release assay (Table I). In other experiments (not shown) we have found no change in the ψMP content of *hisT* tRNA after incubation with homologous extracts. We suspect that the changes in the control incubation were a result of the selective cleavage of UMP residues by the mixture of nuclease activities in the crude cell extracts.

Defective Enzyme Activity in a Temperature-sensitive Mutant—Further evidence that *hisT* mutants are altered in a protein responsible for the formation of pseudouridine residues has been obtained from the study of a temperature-sensitive *hisT* mutant. This mutant, TA263, is subject to normal regulation of the histidine operon when it is grown at 30°, but is derepressed 2- to 3-fold at 37° and 8-fold at 42° (11). If the *hisT* gene product is an enzyme that catalyzes formation of tRNA pseudouridine residues, then the enzyme activity itself should be temperature-sensitive in extracts from strain TA263.

This has proven to be the case when the activity is measured by the tritium release assay (Fig. 9). When the extracts were heated at 60°, the activity from strain TA263 was inactivated 6 to 7 times as rapidly as the activity in wild type extracts.

DISCUSSION

Despite a variety of early conflicting evidence (reviewed in Ref. 26), it is now abundantly clear that the pseudouridine modifications in tRNA occur after assembly of the tRNA chain (27, 28), as is the case with all other known tRNA modification reactions.

The tRNA species which accumulate in *hisT* mutants of *S. typhimurium* provide a natural source of unmodified loci for a specific group of pseudouridine modifications. By the use of *hisT* tRNA, tritiated specifically at the C_5 pyrimidine residues, the tritium release assay provides a convenient and rapid measure of at least a partial reaction involved in pseudouridine formation. The specificity of this assay is implicit from the following considerations. (*a*) Tritium release occurs only when the tRNA from *hisT* cells is treated with wild type extracts (Fig. 2). No release is found when wild type tRNA is used, since all potential sites are already modified. No release is obtained with extracts

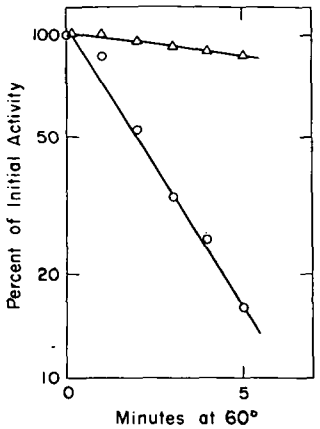


FIG. 9. Temperature sensitivity of wild type and *hisT* extracts. Tubes containing 30 μg of enzyme protein in 100 μl of cell extract were warmed in a 60° bath for the intervals shown and then were immediately chilled in an ice bath. The enzymatic activity was then measured by the tritium release assay, as described under "Materials and Methods." The activity of the untreated extract from the *hisT* mutant, TA263, was only 60% of the activity of the untreated wild type extract. The values shown are normalized to 100% activity for each untreated extract.

from *hisT* cells, since they lack the enzyme. (*b*) The replacement of pseudouridine residues by uridine residues in the anticodon region of $tRNA^{His}$ (13) and $tRNA^{Leu}$ (29) of *hisT* mutants has been established by sequencing. In addition, the chromatographic properties of other species of tRNA are altered in *hisT* mutants, notably those of $tRNA^{Tyr}$ (13) and $tRNA^{Lys}$ (Footnote 2) and the major (13) and minor species of $tRNA^{Leu}$.[3] This pattern is consistent with the known presence of pseudouridine in this position in these tRNA species. (*c*) Although other modification reactions would be measured by the tritium release assay, *e.g.* the formation of 5-methylated or other 5-substituted pyrimidine derivatives (30), it is unlikely that such reactions contribute to the tritium release to any significant extent. This conclusion is based on several facts. First, no additional cofactors were necessary for tritium release, as might be expected for other modification reactions. Second, release of tritium by wild type extracts took place only with *hisT* tRNA as substrate; wild type tRNA, labeled in the same manner, did not release tritium upon treatment with wild type extract. Finally, there is no evidence that *hisT* tRNA has defects in modification other than the absence of certain pseudouridine residues (13).

This assay system may also be suitable for characterizing the pseudouridine modification enzymes of other organisms, particularly by employing heterologous reactions, in which the labeled tRNA is derived from organisms of low pseudouridine content. Such heterologous reactions are readily demonstrable with the tRNA methylation enzymes (31, 32).

Several insights into the enzyme mechanism can be derived, even with the limited information available at this time. (*a*) The enzyme described here is not responsible for the pseudouridine modification in all regions of the tRNA molecule. This is evident from the fact that *hisT* mutants, which lack this activity, still produce tRNA with pseudouridine in the TψCPu loop (13). This residue therefore must be modified independently, presumably by a second enzyme which may act on a tRNA precursor species (33). It is not known whether the pseudouridine residues of ribosomal RNA (34, 35) are formed by still another enzyme or not. For these reasons, we propose the designation tRNA pseudouridine synthetase I for the enzyme described here, which is absent or defective in *hisT* mutants, and tRNA pseudouridine synthetase II for the activity implicated in the TψCPu sequence. (*b*) Although there has been some speculation that the formation of pseudouridine could derive from a cytidine residue (see Ref. 24, p. 310), the most likely precursor would appear to be an unmodified uridine residue. This likelihood is implicit in the normal base-pairing relationships of the pseudouridine residues in the anticodon stem (10, 13), and in the fact that *hisT* tRNA contains uridine, rather than cytidine residues, in place of the missing pseudouridine residues of wild type tRNA (13). (*c*) The reaction mechanism probably does not involve loss of a hydrogen atom from carbon 6, since *hisT* tRNA tritiated at carbon 6 does not react with wild type enzyme (Fig. 2).

Breitman (20) has used this type of tritium release assay to characterize the enzyme, pseudouridylate synthetase, which catalyzes the formation of ψMP from uracil and ribose-5-P, in extracts from various strains of *E. coli*. We have been unable to detect this activity in our wild type strain of *S. typhimurium* (LT-2) by direct assay. In addition, strain LT-2 is unable to grow on pseudouridine as a carbon source, nor can the growth of *pyr* auxotrophs of LT-2 be supported by pseudouridine. These findings, and the lack of a specific tRNA requirement in the pseudouridylate synthetase reaction, clearly show that it is a distinct enzyme from the tRNA pseudouridine synthetase described here.

The biological effects of the *hisT* mutation are uniquely pleiotropic. The lack of the pseudouridine residues in the anticodon region of $tRNA^{His}$ in some manner affects the production of the histidine biosynthetic enzymes. The same *hisT* mutant is also altered in the regulation of the *leu* and *ile* operons.[3] *HisT* mutants also possess an altered $tRNA^{Lys}$ and are resistant to inhibition by the lysine analog, 2-*S*-aminoethylcysteine.[2] The manner in which the lack of pseudouridine modifications can lead to these regulatory defects remains unclear, but is obviously of great interest.

Finally, the analysis of a temperature-sensitive *hisT* mutant suggests that the *hisT* gene codes for the modification enzyme rather than for a protein which regulates the activity or synthesis of the modification enzyme.

[2] R. Cortese, R. Landsberg, and B. N. Ames, unpublished observations.

[3] R. Cortese, R. Von der Haar, R. Landsberg, H. E. Umbarger, and B. N. Ames, manuscript in preparation.

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